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Enzymes Involved in Anaerobic Respiration Appear To Play a Role in *Actinobacillus pleuropneumoniae* Virulence

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Actinobacillus pleuropneumoniae*, the etiological agent of porcine pleuropneumonia, is able to survive on respiratory epithelia, in tonsils, and in the anaerobic environment of encapsulated sequesters. It was previously demonstrated that a deletion of the anaerobic dimethyl sulfoxide reductase gene (*dmsA*) results in attenuation in acute disease (N. Baltes, S. Kyaw, I. Hennig-Pauka, and G. F. Gerlach, Infect. Immun. 71:6784–6792, 2003). In the present study, using two-dimensional polyacrylamide gel electrophoresis and quadrupole time-of-flight mass spectrometry, we identified an aspartate ammonia-lyase (AspA) which is upregulated upon induction with bronchoalveolar lavage fluid (BALF). This enzyme is involved in the production of fumarate, an alternative electron acceptor under anaerobic conditions. The coding gene (*aspA*) was cloned and shown to be present in all *A. pleuropneumoniae* serotype reference strains. The transcriptional start point was identified downstream of a putative FNR binding motif, and BALF-dependent activation of *aspA* was confirmed by construction of an isogenic *A. pleuropneumoniae* mutant carrying a chromosomal *aspA*::*luxAB* transcriptional fusion. Two *aspA* deletion mutants, *A. pleuropneumoniae* Δ*aspA* and *A. pleuropneumoniae* Δ*aspA*Δ*dmsA*, were constructed, both showing reduced growth under anaerobic conditions in vitro. Pigs challenged with either of the two mutants in an aerosol infection model showed a lower lung lesion score than that of the *A. pleuropneumoniae* wild-type (wt) controls. Pigs challenged with *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* had a significantly lower clinical score, and this mutant was rarely reisolated from unaltered lung tissue; in contrast, *A. pleuropneumoniae* Δ*aspA* and the *A. pleuropneumoniae* wt were consistently reisolated in high numbers. These results suggest that enzymes involved in anaerobic respiration are necessary for the pathogen's ability to persist on respiratory tract epithelium and play an important role in *A. pleuropneumoniae* pathogenesis.

Actinobacillus pleuropneumoniae causes porcine pleuropneumonia, a disease occurring worldwide and causing significant economic losses (13). A major problem in controlling the disease is the pathogen's ability to persist on respiratory tract epithelia, in tonsils, and in sequestered lung tissue for weeks or months after infection, resulting in clinically healthy carrier animals (9, 13, 20). Since the oxygen level in necrotic tissue is reduced, survival of bacteria requires strategies for anaerobic respiration using alternative electron acceptors. In a previous study, an *A. pleuropneumoniae* dimethyl sulfoxide (DMSO) reductase was identified, and an isogenic deletion mutant (*A. pleuropneumoniae* Δ*dmsA*) was shown to be attenuated in acute disease (2); however, this mutant and the parent strain were indistinguishable with respect to the formation of lung lesions and the ability to persist, thereby implying that additional alternative electron acceptors might facilitate anaerobic respiration in the absence of DMSO reductase.

In *Escherichia coli*, fumarate can serve as a terminal electron acceptor under anaerobic conditions (48). Fumarate is produced from oxaloacetate via malate involving malate dehydrogenase or via aspartate by aspartate aminotransferase and aspartate ammonia-lyase (aspartase) (24, 30). Aspartase has been extensively studied in *E. coli*, where it is regulated by the global anaerobic regulator FNR (25, 51) and is increased under anaerobic conditions (19, 25), whereas the malate dehydrogenase is downregulated (10). Therefore it was suggested that, under anaerobic conditions, fumarate production is mediated by the aspartase pathway rather than by malate dehydrogenase (10, 19, 24). Besides its role in anaerobic respiration, aspartase is required for utilization of L-glutamate and L-asparagine as carbon sources (26, 29).

An aspartate ammonia-lyase has been described in several other gram-negative organisms including *Klebsiella aerogenes* (47), *Salmonella enterica* serovar Typhimurium (27), *Pseudomonas* species (43), *Eikenella corrodens* (39), and *Haemophilus influenzae* (41), and the *H. influenzae* aspartase was shown to possess the additional function of binding plasminogen (41).

In the present study, we compared protein expression patterns of *A. pleuropneumoniae* cultured in the presence of porcine bronchoalveolar lavage fluid (BALF) with the protein pattern of control cultures. We identified an *A. pleuropneumoniae* aspartate ammonia-lyase which is reproducibly upregulated in the ex vivo model. The coding gene (*aspA*) was cloned, and an isogenic deletion mutant (*A. pleuropneumoniae* Δ*aspA*) and a double mutant (*A. pleuropneumoniae* Δ*aspA*Δ*dmsA*) were constructed. Both mutants were characterized in vitro as well as in vivo by using aerosol infection experiments.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains, plasmids, and primers used in this work are listed in Table 1. *E. coli* strains were cultured in Luria-Bertani medium supplemented with the appropriate antibiotic (ampi-

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Characteristic(s)	Source and/or reference
Strains		
E. coli DH5αF′	F′ *endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ80d*lac*Δ(*lacZ*)*M15*]	36
E. coli β2155	*thrB1004 pro thi strA hsdS lacZΔM15* (F′ *lacΔM15 laqI*^{q} traD36 *proA*$^+$ *proB*$^+$)Δ*dapA*::*erm* (Erm^r) *recA*::*RPA-2-tet* (Tc^r)::Mu-km (Km^r) λ *pir*	11
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara leu*) *7697 galU galK rpsL* (Str^r) *endA1 nupG*	TOPO TA
A. pleuropneumoniae AP76 (wt)	*A. pleuropneumoniae* serotype 7 strain[b]	1
A. pleuropneumoniae Δ*aspA*	Unmarked *aspA*-negative knockout mutant of *A. pleuropneumoniae* AP76	This work
A. pleuropneumoniae Δ*aspA*Δ*dmsA*	Unmarked *aspA*- and *dmsA*-negative double-knockout mutant of *A. pleuropneumoniae* AP76	This work
A. pleuropneumoniae Δ*asp*A::*lux*AB	Unmarked *A. pleuropneumoniae* mutant carrying *lux*AB as transcriptional fusion in the *asp*A gene	This work
ATCC 27088	*A. pleuropneumoniae* serotype 1 reference strain	ATCC
ATCC 27089	*A. pleuropneumoniae* serotype 2 reference strain	ATCC
ATCC 27090	*A. pleuropneumoniae* serotype 3 reference strain	ATCC
ATCC 33378	*A. pleuropneumoniae* serotype 4 reference strain	ATCC
ATCC 33377	*A. pleuropneumoniae* serotype 5A reference strain	ATCC
ATCC 33590	*A. pleuropneumoniae* serotype 6 reference strain	ATCC
WF83	*A. pleuropneumoniae* serotype 7 reference strain	Rosendal (37)
405	*A. pleuropneumoniae* serotype 8 reference strain	Nielsen
CVJ13261	*A. pleuropneumoniae* serotype 9 reference strain	Nielsen
D13039	*A. pleuropneumoniae* serotype 10 reference strain	Nielsen
56153	*A. pleuropneumoniae* serotype 11 reference strain	Nielsen
8329	*A. pleuropneumoniae* serotype 12 reference strain	Nielsen
N273	*A. pleuropneumoniae* serotype 13 reference strain	Angen (32)
3906	*A. pleuropneumoniae* serotype 14 reference strain	Angen (32)
HS143	*A. pleuropneumoniae* serotype 15 reference strain	Angen (6)
Plasmids		
pCR2.1-TOPO	*E. coli* cloning vector for fast and efficient cloning of *Taq* polymerase-amplified PCR products	Invitrogen
pBluescript SKΔKpnI	*E. coli* cloning vector lacking a KpnI site and carrying an ampicillin resistance determinant	Stratagene Europe, Amsterdam, The Netherlands
pAS810	PCR product obtained with primers oAS7 and oAS8 cloned into pCR2.1-TOPO	This work
pAS110	EcoRI fragment from pAS810 containing *aspA* ligated into EcoRI-restricted pBluescript SKΔKpnI	This work
pAS112	pAS110 was restricted with Acc651 and SnaBI; the 5′ overhang generated by Acc651 was filled in by T4 polymerase, and the plasmid was religated	This work
pBMK1	Transconjugation vector based on pBlueskript SK with *mobRP4*, a polycloning site, Km^r, and a transcriptional fusion of the *omlA* promoter with the *sacB* gene	34
pAS610	Ligation of a SalI-XbaI fragment from pAS112 into pBMK1 cut with SalI and XbaI	This work
pAS620	Ligation of a SalI-XbaI fragment from pAS110 into pBMK1 cut with SalI and XbaI	This work
pDM800	Ligation of Δ*dmsA* into pBMK1	2
pSB417	pUC18Sfi containing a promoterless *luxCDABE* EcoR1 cassette	50
pEMOC2	Transconjugation vector based on pBluescript SK with *mobRP4*, a polycloning site, Cm^r, and transcriptional fusion of the *omlA* promoter with the *sacB* gene	3
pASLux101	Ligation of *luxAB* (PCR) product obtained with oASL1 and oASL2, restricted with Acc65I-SnaBI) into Acc65I-SnaBI, restricted pAS110	This work
pASLux700	Ligation of a PspOMI-NotI fragment from pASLux101 into pEMOC2	This work
Primers		
oAS1	GGTCTAGAGCGATTGTAGCWGCTTGTGATG (upstream primer with an internal XbaI site comprising positions 219 to 241 of the *aspA* ORF)	This work
oAS2	CGTCTAGACGTAAGTTCACACAAGCATTGG (downstream primer with an internal XbaI site comprising positions 1135 to 1158 of the *aspA* ORF)	This work
oAS4	CTTCCGCTTTTGCTTCGAAG (downstream primer comprising positions 760 to 779 of the *aspA* ORF)	This work
oAS5	CTTCGAAGCAAAAGCGGAAG (upstream primer comprising positions 760 to 779 of the *aspA* ORF)	This work
oAS6	AGGTAAACCGGTTACTTCCG (downstream primer comprising positions 518 to 537 of the *aspA* ORF)	This work
oAS7	GAGGTGAATTTATGAGTAATGTACGTG (upstream primer comprising positions 11 to 18 of the *aspA* ORF)	This work
oAS8	AAWTACTCTTCTTCAGTAAATTTCGCC (downstream primer comprising positions 1405 to 1432 of the *aspA* ORF)	This work
oAS15	TTGTTCGCGGTATGGTAATGGTAAAAA (upstream primer comprising positions 136 to 163 of the *aspA* ORF)	This work
oAS16	CCGCATCTTGTAACTGAGTA (downstream primer comprising positions 564 to 583 of the *aspA* ORF)	This work

Continued on following page

TABLE 1—*Continued*

Strain, plasmid, or primer	Characteristic(s)	Source and/or reference
oDMSAdel1	TTGAAATATCCGATGAAACGT (downstream primer comprising positions 317 to 338 of the *dmsA* homologue)	2
oDMSAdel2	TCATATTGGCGACATAAGCAT (upstream primer comprising positions 1583 to 1604 of the *dmsA* homologue)	2
5′ RACE Abridged Anchor primer	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG (primer binding to the Oligo (dC) tail in the 5′ RACE system)	Invitrogen
oASL1	GTAAGGTACCATAGGGACTCTCTATGAAATTTGGAAAC (upstream primer with an internal Acc65I site for amplification and cloning of *luxAB*)	This work
oASL2	TCATTACGTAGCTGCAACTCGAAATCTATTAGG (downstream primer with an internal SnaBI site for amplification and cloning of *luxAB*)	This work

[a] TOPO TA, TOPO TA cloning kit (Invitrogen); ATCC, American Type Culture Collection, Manassas, Va.; Rosendal, S. Rosendal, University of Guelph, Guelph, Ontario, Canada (37); Nielsen, R. Nielsen, State Veterinary Serum Laboratory, Copenhagen, Denmark; Angen, O. Angen, Danish Institute for Food and Veterinary Research, Copenhagen, Denmark.

[b] Strain AP76 was kindly provided by the Western College of Veterinary Medicine, Saskatoon, Canada.

cillin [100 μg/ml]); for cultivation of *E. coli* β2155 (Δ*dapA*), diaminopimelic acid (1 mM; Sigma Chemical Company, Deisenhofen, Germany) was added. *A. pleuropneumoniae* serotype 7 parent and mutant strains were cultured at 37°C in PPLO medium (Difco GmbH, Augsburg, Germany) supplemented with NAD (10 μg/ml; Merck AG, Darmstadt, Germany), L-glutamine (100 μg/ml; Serva, Heidelberg, Germany), L-cysteine hydrochloride (260 μg/ml; Sigma), L-cystine dihydrochloride (10 μg/ml; Sigma), dextrose (1 mg/ml), and Tween 80 (0.1%). To investigate the change in protein profiles upon induction by BALF, a 100-ml *A. pleuropneumoniae* culture was grown with shaking at 200 rpm to an optical density at 660 nm (OD_{660}) of 0.3. This culture was split into 20-ml aliquots, and an equal volume of freshly thawed BALF or, for control cultures, an equal volume of NaCl (150 mM) was added to the culture medium. Cultures with BALF and aerobic control cultures were further incubated with shaking for 1 h, growing to an OD_{660} of approximately 0.6. Anaerobic cultures used for proteome analysis and determination of aspartase activity were placed into an anaerobic jar without shaking at 37°C for 3 h after the addition of NaCl (150 mM), reaching an OD_{660} of approximately 0.6. For investigation of DmsA expression under anaerobic conditions and plasminogen binding, 20- and 100-ml *A. pleuropneumoniae* cultures, respectively, were grown to an OD_{660} of 0.3 in a shaking incubator followed by incubation in an anaerobic jar without shaking for 3 h. Cells were harvested by centrifugation, washed once in 50 mM Tris-HCl (pH 7.2), resuspended in 50 mM Tris-HCl (pH 7.2), and stored at −70°C. To compare the ability of the different strains to grow under anaerobic conditions, 100 ml of supplemented PPLO medium was preincubated in an anaerobic chamber for at least 48 h, inoculated with a single colony, and further incubated at 37°C for 16 h. Since all *A. pleuropneumoniae* strains used in this study showed heavy clumping under anaerobic conditions, bacterial growth was determined as dry pellet weight; bacteria were harvested by centrifugation, and the pellet was dried at 80°C for 24 h. Statistical analysis of pellet weights was performed by using the Student's *t* test.

Preparation of whole-cell lysates, 2D-PAGE, and quadrupole time-of-flight mass spectrometry (Q-TOF MS). Cells were ruptured by using a Mini-Bead Beater (BioSpec Products, Inc.) three times for 3 min followed by sonication (Sonifier cell disruptor; SmithKline Corporation) twice with 30 pulses on ice. Ruptured cells were treated with Benzonase (Merck Biosciences, Schwalbach, Germany) for 10 min at 37°C. Unbroken cells were removed by centrifugation at 16,000 × *g* in a benchtop centrifuge for 10 min. Protein concentration was determined with a MicroBC assay (Uptima Interchim, Montlucon Cedex, France). For two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), 500 μg of protein was precipitated overnight with trichloroacetic acid (10% final concentration) and pelleted by centrifugation at 16,000 × *g* in a benchtop centrifuge. The pellets were washed once with pure acetone and solubilized in 340 μl of isoelectric focusing solution consisting of 7 M urea, 2 M thiourea (Sigma), 1% (wt/vol) ASB 14 (Merck), 0.5% (vol/vol) Triton X-100, 40 mM Tris base, 30 mM dithiothreitol, and 0.5% IPG buffer (pH 4 to 7; Amersham Biosciences AB, Uppsala, Sweden). Insoluble material was removed by centrifugation at 16,000 × *g* for 5 min in a benchtop centrifuge. Immobiline DryStrips (18 cm, pH 4 to 7; Amersham) were first rehydrated with 340 μl of the isoelectric focusing solution containing the sample (rehydration loading) for 11 h and subsequently focused by using an Ettan IPGphor (Amersham) for 11 h in the following series of time blocks with increasing voltage: 2 h at 100 V, 1 h at 500 V, 1 h at 1,000 V, 2 h at an 8,000-V gradient, and 5 h at 8,000 V. For the second dimension, the strips were equilibrated twice for 10 min by rocking in a solution of 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate (SDS), and 10 mg of dithiothreitol/ml. Separation of proteins in the second dimension was achieved by discontinuous SDS-PAGE (10.8% acrylamide and 0.29% bisacrylamide). Gels were run at 15°C and 80 V for 15 min followed by 200 V for 3.5 h. Proteins were visualized by staining the gels overnight with colloidal Coomassie G-250 (31). For each growth condition, at least three preparations from independent cultures were used to generate 2D gels for spot comparison. Spots present only in gels derived from bacteria induced with BALF and spots which showed increased intensity in all gels from BALF-induced cultures compared to control cultures were cut from the gels and treated by using a slightly modified method of Shevchenko et al. (40). Briefly, the gel pieces were washed with water, dehydrated with acetonitrile, rehydrated with 100 mM NH_4HCO_3, and, prior to trypsin (sequencing grade; Promega, Mannheim, Germany) digestion again dehydrated by acetonitrile. Peptides were extracted by 25 mM NH_4HCO_3 followed by acetonitrile and by 5% formic acid followed by acetonitrile. Extracted peptides were purified by using ZipTip C_{18} microcolumns (Millipore, Billerica, Mass.). Peptide sequences were determined from tandem mass spectrometry (MS/MS) fragmentation data recorded on an ESI Q-TOF II mass spectrometer (Micromass, Milford, Mass.). Proteins were identified by using the program Sonar MS/MS (Proteometrics, New York, N.Y.) by searching against either the genome of *H. influenzae* (GenBank accession no. NC_000907) or the National Center for Biotechnology Information bacterial genomes (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

Manipulation of DNA. DNA-modifying enzymes were purchased from New England Biolabs (Bad Schwalbach, Germany) and used according to the manufacturer's instructions. *Taq* polymerase was purchased from Gibco-BR Life Technologies (Karlsruhe, Germany). Chromosomal DNA for PCR and Southern blotting as well as plasmid DNA were prepared by standard protocols (38). PCR, Southern blotting, transformation, and gel electrophoresis were done by standard procedures (38), and pulsed-field gel electrophoresis (PFGE) was performed as described previously (33).

Cloning of the *A. pleuropneumoniae aspA* gene and construction of unmarked isogenic mutants. For cloning of the *A. pleuropneumoniae* aspartase, sequence information from the *aspA* genes from *H. influenzae* (GenBank accession no. U32735) and *Pasteurella multocida* (GenBank accession no. AE006151) were compared, and primers oAS1 and oAS2, which comprise conserved regions of these genes, were constructed (Table 1). The PCR product generated with these primers using *A. pleuropneumoniae* wild-type (wt) chromosomal DNA as a template was sequenced (SeqLab, Göttingen, Germany). The nucleotide sequence obtained was used to query the public DNA and protein databases by using BLASTN 2.2.5. The retrieved sequences (GenBank accession no. NC_003998, NC_004130, and NC_004427) were used to construct primers oAS7 and oAS8 (Table 1). The PCR product obtained with these primers was cloned into TOPO 2.1, and the resulting plasmid was restricted with EcoRI and ligated into pBluescript SKΔKpnI (Table 1) restricted with EcoRI, resulting in plasmid pAS110 (Table 1). To construct conjugation plasmid pAS610 (Table 1) the following steps were required. (i) To create an in-frame deletion, pAS110 was restricted with Acc65I and SnaBI. The 5′ overhang generated by Acc65I was filled in by using T4 DNA polymerase, and the plasmid was religated, resulting in plasmid pAS112 (Table 1). The deletion was characterized by restriction enzyme digest and PCR. (ii) The insert from pAS112 was removed with SalI and XbaI and ligated into the transconjugation vector pBMK1 (34) cut with SalI and XbaI. The resulting plasmid, pAS610, was used in a single-step transconjugation system (34)

to construct the mutant *A. pleuropneumoniae* Δ*aspA*. Plasmid pDM800 (5) was used to introduce a *dmsA* in-frame deletion into *A. pleuropneumoniae* Δ*aspA*. The conjugation, selection, and counterselection procedures were performed as described previously (34). Colonies obtained after counterselection were tested by PCR using primers oAS5 and oAS6 (Table 1) for *A. pleuropneumoniae* Δ*aspA* and oDMSAdel1 and oDMSAdel2 (Table 1) for the double mutant *A. pleuropneumoniae* Δ*aspA*Δ*dmsA*. Colonies with the correct PCR profile were confirmed by Southern blot analysis using the PCR product obtained with primers oAS15 and oAS8 or primers oDMSAdel1 and oDMSAdel, respectively, as a probe. The absence of genomic rearrangements in both mutants was confirmed by PFGE. Expression of DmsA was evaluated by Western blot analysis; aspartase activity was determined as described below.

Complementation of *A. pleuropneumoniae* Δ*aspA*. Conjugation plasmid pAS620 carrying the intact *aspA* gene was constructed by ligating the SalI-XbaI fragment obtained from pAS110 into SalI-XbaI-restricted pBMK1. Conjugation with *A. pleuropneumoniae* Δ*aspA* as recipient strain, selection, and counterselection were performed as described previously (34). Several clones carrying the wild-type gene were tested for aspartase activity as described below.

Determination of the transcriptional start point of *aspA*. The 5′ RACE System for Rapid Amplification of cDNA Ends (version 2.0; Invitrogen, Groningen, The Netherlands) was used. Briefly, RNA from the *A. pleuropneumoniae* wt grown under both aerobic and anaerobic conditions was prepared by using an RNeasy Mini kit from QIAGEN (Hilden, Germany). After DNase treatment (Turbo DNase; Ambion, Austin, Tex.), first-strand DNA was synthesized by using primer oAS16 as an *aspA*-specific primer. RNA was removed by RNase treatment (RNase, DNase free; Roche, Basel, Switzerland) followed by purification of cDNA using a Gene Clean kit (Qbiogene, Heidelberg, Germany). An oligo(dC) tail was added to the 3′ end of the purified cDNA by using terminal deoxynucleotidyl transferase (Invitrogen), and the tailed cDNA was amplified by PCR using 5′ RACE Abridged Anchor primer (Invitrogen) and oAS4 as an *aspA*-specific primer. The PCR products were cloned into pCR2.1-TOPO by using a TOPO TA cloning kit. Six of the resulting clones were sequenced.

Determination of aspartase activity. Aspartase activity was measured spectrophotometrically at 240 nm by determination of fumarate formation (44). The assay buffer contained 3 mM $MgCl_2$, 0.1 M L-aspartate (Sigma), and 0.1 M Tris-HCl (pH 9.0). The reaction was initiated by the addition of cell lysates, and the increase in absorbency at 240 nm was determined (35). Aspartase activity was expressed in units, with 1 U being the amount that converts 1.0 nmol of L-aspartate to fumarate per minute. A molar extinction coefficient of 2,530 M^{-1} cm^{-1} at 240 nm (44) was used to calculate the activity, and statistical analysis was performed by using the Student's *t* test.

Construction of an *A. pleuropneumoniae* mutant carrying a transcriptional fusion of *aspA* and *luxAB*. The *luxAB* genes coding for the luciferase of *Photorhabdus luminescens* were amplified from plasmid pSB417 (50), kindly provided by M. K. Winson, by using primers oASL1 and oASL2. Primer oASL1 contains a stop codon, TAG, for *aspA* at positions 12 to 14, overlapping with a Shine-Dalgarno consensus sequence for *luxAB*. The PCR product was restricted with Acc65I and SnaBI and ligated into Acc65I-SnaBI-restricted pAS110, resulting in plasmid pASLux101 carrying the *aspA* gene with a *luxAB* insertion. To construct conjugation vector pASLux700, the PspOMI-NotI fragment from pASLux101 was ligated into PspOMI-NotI-restricted pEMOC2. Conjugation plasmid pASLux700 was sequenced to confirm in-frame insertion of *luxAB* into *aspA*. By using pASLux700 and *A. pleuropneumoniae* AP76 in the single-step transconjugation system, *A. pleuropneumoniae* Δ*aspA*::*luxAB* was constructed.

Luciferase assay. *A. pleuropneumoniae* Δ*aspA*::*luxAB* was grown as described above for induction with BALF, with the exception of the anaerobic control culture. The anaerobic incubation was done for only 1 h to allow analysis in parallel with aerobic control and BALF-induced cultures. Prior to luciferase analysis, the anaerobic culture was vigorously shaken for 30 s and left standing for 10 min in normal atmosphere to provide sufficient oxygen for the luciferase reaction to occur. Briefly, 2.5 ml of each culture was transferred to a 24-well plate and mixed with 5 μl of 1% *N*-decyl-aldehyde (Sigma). An X-ray film was then exposed to the 24-well plate for 3 min.

Investigation of plasminogen-binding capability of *A. pleuropneumoniae* aspartase. Porcine plasminogen was purified from serum by using Lysine Sepharose 4B (Amersham) and subsequently coupled to CNBr-activated Sepharose (Amersham) according to the manufacturer's instructions. Cell lysates of the *A. pleuropneumoniae* wt and Δ*aspA* were used for affinity chromatography to purify plasminogen-binding proteins as described previously by Sjöström et al. (41). Eluted material from the affinity column was analyzed by discontinuous SDS-PAGE (10.8% acrylamide and 0.29% bisacrylamide).

Western blot analysis. *A. pleuropneumoniae* whole-cell lysates were analyzed by discontinuous SDS-PAGE (10.8% acrylamide and 0.29% bisacrylamide) and Western blotting using a Protean II Minigel system (Bio-Rad) as described previously (16). The serum directed against DmsA had been raised in rabbits as described previously (2).

BALF. During a previous virulence study, bronchoalveolar lavage with saline (150 mM) was performed on pigs prior to infection and on day 7 and day 21 after experimental infection with *A. pleuropneumoniae* AP76 as described previously (4). For induction of differential protein expression of *A. pleuropneumoniae*, BALF from eight animals was pooled and centrifuged (6,000 × *g*, 10 min) to remove cells and bacteria and stored at −70°C for a maximum of 12 months until use. The absence of viable bacteria in BALF was determined by culturing on Columbia sheep blood agar plates and supplemented PPLO agar plates.

Determination of urease activity of *A. pleuropneumoniae* colonies. Bacterial cultures on supplemented PPLO agar were overlaid with 0.5% agarose containing 0.3 M urea and 0.01% phenol red (Sigma). The color of the colonies was assessed after 1 min. Urease-positive colonies turned red, whereas urease-negative colonies turned yellow.

Virulence studies. Virulence of *A. pleuropneumoniae* Δ*aspA* and *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* was assessed in an aerosol infection model as previously described (4). *A. pleuropneumoniae*-free and clinically healthy pigs 7 to 9 weeks of age were randomly assigned to the different groups and cared for in accordance with the principles outlined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series, no. 123 [http://conventions.coe.int/treaty/EN/Menuprincipal.htm]). Clinical examinations were performed daily or as needed. Body temperature and clinical symptoms were recorded daily for each individual pig. A clinical scoring system based on the directive from the European Pharmacopoeia for the testing of *A. pleuropneumoniae* vaccines (porcine actinobacillosis vaccine [inactivated]) was employed to assess the clinical condition of each individual animal as follows. A score of 1 was given for each symptom including the occurrence of coughing, dyspnea, and vomitus, resulting in a minimum clinical score of 0 and a maximum score of 3 per day; the added daily clinical scores of days 1 to 7 were designated as the total clinical score. Statistical analysis of the total clinical score was performed by using the Student's *t* test. In order to confirm the absence of *A. pleuropneumoniae*-specific antibodies, blood samples were taken 1 week prior to infection; blood samples on day 7 and day 21 post-infection were taken to determine the serological response to challenge with the different *A. pleuropneumoniae* strains. Postmortem analysis as well as bacteriological and serological examinations were performed as described previously (4). Briefly, lung lesion scores were determined as described previously by Hannan et al. (21) and statistically analyzed using the Wilcoxon test. The bacteriological examination included surface swabs of affected and unaffected lung tissue, palatine tonsils, bronchial lymph nodes, and heart muscle on Columbia sheep blood agar and selective meat-blood agar (23). Several individual *A. pleuropneumoniae*-like colonies were subcultured on supplemented PPLO agar and confirmed by urease assay and PCR analyses using primers oAS5 and oAS6 or oDMSAdel1 and oDMSAdel2. Lung tissues were immersion fixed in formalin and embedded in paraffin, and thin sections (5 μm) were stained with hematoxylin and eosin.

RESULTS

Identification and characterization of the *A. pleuropneumoniae* aspartate ammonia-lyase. The proteomes of *A. pleuropneumoniae* AP76 grown under control conditions and grown with the addition of BALF were compared by 2D PAGE (Fig. 1). Protein spots that showed increased intensity in the proteome of bacteria grown with the addition of BALF were analyzed by using Q-TOF MS. Two of these proteins of approximately 50 kDa and with an isoelectric point (IP) of 5.2 and 5.25, respectively, showed high homology to the aspartate ammonia-lyase of *H. influenzae* (AAC22191.1). Using primers oAS1 and oAS2, we amplified a 939-bp fragment from chromosomal DNA of *A. pleuropneumoniae* AP76. Comparison of sequencing results from this fragment with public DNA databases revealed a 1,506-bp open reading frame (ORF) encoding a protein with a calculated molecular mass of 51.7 kDa and a calculated isoelectric point of 5.14 in the unfinished genomes of *A. pleuropneumoniae* (GenBank accession no. NC_003998, NC_004130, and NC_004427), and PCR analyses using primers oAS7 and oAS8 revealed the presence of the *aspA* gene in

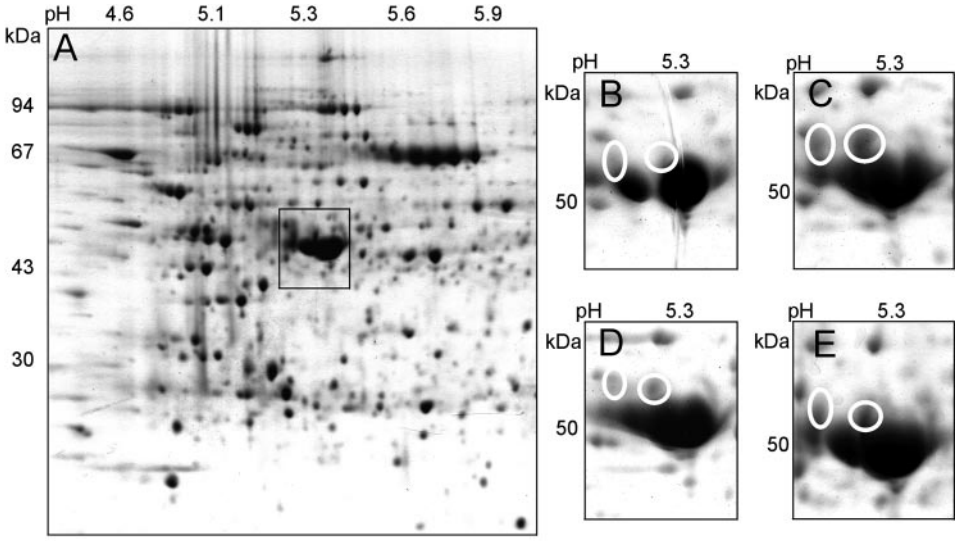

FIG. 1. Two-dimensional gel electrophoresis of *A. pleuropneumoniae* whole-cell lysates. (A) Gel loaded with 500 μg of protein of the *A. pleuropneumoniae* wt grown with the addition of BALF from day 21 after infection, focused on linear Immobiline DryStrips (pH 4 to 7). The boxed area is shown enlarged in B to E, representing the following growth conditions: (B) aerobic control culture, (C) anaerobic culture, (D) addition of BALF collected from uninfected pigs, and (E) addition of BALF collected on day 21 after infection with *A. pleuropneumoniae* AP76. White circles indicate proteins identified as aspartate ammonia-lyase. The experiments were performed in triplicate with lysates from independent cultures; results similar to the ones shown here were obtained in each experiment.

A. pleuropneumoniae serotype reference strains 1 to 15 (data not shown); furthermore, database analysis showed that the *A. pleuropneumoniae* protein is 88.6% identical to the aspartate ammonia-lyase of *H. influenzae*, which had been found to possess plasminogen-binding activity (41).

A Shine-Dalgarno consensus sequence was located 7 bp upstream of the start codon (Fig. 2). Using the 5′ RACE kit, we obtained PCR products from anaerobically grown bacteria but not from an aerobic control culture. Sequencing results from these PCR products indicated that the transcriptional start point is located 40 bp upstream of the start codon, preceded by a putative promoter sequence, TATGAT. Furthermore, a single putative FNR binding domain (GTGAT-$N_1N_2N_3N_4$-ATCAC) (18) is located 35 bases upstream of the transcriptional start site.

The activity of the aspartase in whole-cell lysates differed by a factor of 1.5 to 2 depending on the growth conditions (Table 2). Aspartase activity was significantly increased under anaerobic conditions and by the addition of BALF compared to aerobic control conditions ($P < 0.02$); however, induction upon growth under anaerobic conditions was significantly stronger than that obtained by the addition of BALF ($P < 0.01$). The induction of *aspA* transcription by BALF was confirmed by the analysis of *A. pleuropneumoniae* Δ*aspA*::*luxAB*, a mutant constructed to express luciferase in transcriptional fusion with the truncated *aspA* gene on the chromosome; this mutant clearly showed enhanced transcription of *aspA* upon induction by both anaerobic conditions and the addition of BALF (Fig. 3). No plasminogen-binding activity, as described for the *H. influenzae* aspartase (41), could be detected in *A. pleuropneumoniae* cell lysates.

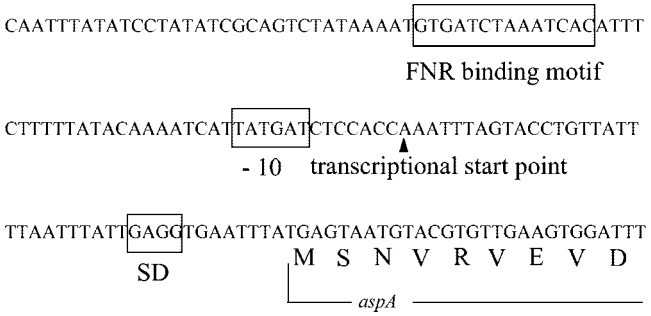

FIG. 2. Promoter region of the *aspA* gene of the *A. pleuropneumoniae* wt. SD indicates the position of the Shine-Dalgarno consensus sequence.

Functional characterization of isogenic mutants *A. pleuropneumoniae* Δ*aspA* and *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* in vitro. A 57-bp in-frame deletion was introduced into *A. pleuropneumoniae* AP76 via conjugation using plasmid pAS610 followed by sucrose counterselection as described previously (45). The resulting *A. pleuropneumoniae* Δ*aspA* mutant was verified by using PCR, Southern blot, and PFGE analyses (data

TABLE 2. Aspartase activity in cell lysates grown under different conditions

Growth condition	Aspartase activity (U/mg of cell lysate)[a]
Aerobic control	395 ± 92
Anaerobic culture	877 ± 105
BALF prior to infection	652 ± 46[b]
BALF day 7 after infection	586 ± 40[b]
BALF day 21 after infection	599 ± 40[b]

[a] Results from six independent experiments per growth condition are shown.
[b] Statistically significant difference (determined by Student's *t* test). Compared to both aerobic and anaerobic controls ($P < 0.02$).

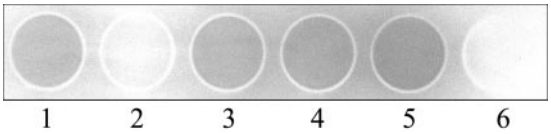

FIG. 3. Luciferase assay of *A. pleuropneumoniae* Δ*aspA*::*luxAB*. Cells were cultured under anaerobic (1) and aerobic (2) conditions and induced by the addition of BALF from animals prior to infection (3) and day 7 (4) and day 21 (5) after infection with *A. pleuropneumoniae* AP76; lane 6 contains medium only. For each culture, 2.5 ml was exposed to an X-ray film for 3 min.

not shown). *A. pleuropneumoniae* Δ*aspA* was then used to construct the double mutant *A. pleuropneumoniae* Δ*aspA*Δ*dmsA*, carrying deletions in the *aspA* gene and the *dmsA* gene; the mutant was verified as described above (data not shown). Both mutants lacked detectable aspartase activity (Fig. 4). Aspartase activity could be restored by reintroducing the complete *aspA* gene into the chromosome (Fig. 4). The *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* mutant showed no expression of DmsA in Western blot analyses (data not shown).

Under anaerobic growth conditions, both *A. pleuropneumoniae* Δ*aspA* and *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* showed significantly decreased growth over a 16-h incubation period compared to both the *A. pleuropneumoniae* wt and *A. pleuropneumoniae* Δ*dmsA* ($P < 0.01$); no difference was apparent between the two aspartase-negative mutants (Fig. 5). In contrast, growth of *A. pleuropneumoniae* wt and mutant strains was undistinguishable under aerobic conditions (data not shown).

Virulence studies. In a first experiment, *A. pleuropneumoniae* Δ*aspA* was used in an aerosol infection model and compared to the parental strain, the *A. pleuropneumoniae* wt. The challenge doses were 3.6×10^4 bacteria (aerosolized for four pigs) for the group challenged with the *A. pleuropneumoniae* wt and 2.2×10^4 bacteria for the group challenged with the *A. pleuropneumoniae* Δ*aspA*. The animals were sacrificed on day 21 postinfection. In a second aerosol infection experiment with higher challenge doses, groups of pigs were infected with mutant strain *A. pleuropneumoniae* Δ*aspA* or *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* (16.4×10^4 bacteria and 11.1×10^4 bacteria for four pigs, respectively) and compared to a control group infected with the *A. pleuropneumoniae* wt (6.8×10^4 bacteria for four pigs). Pigs were sacrificed on day 7 except for three randomly assigned animals in the group challenged with *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* and four randomly assigned animals in the control group, which were sacrificed on day 21. Infection with the *A. pleuropneumoniae* wt and both mutants led to an increase in body temperature above 40°C in 38 of the 44 challenged pigs, with no apparent difference observed between the challenge groups (data not shown). However, dyspnea was observed longer in pigs challenged with the *A. pleuropneumoniae* wt compared to either one of the mutant strains (Fig. 6A). Animals infected with *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* showed a significantly lower clinical score ($P < 0.01$) than animals infected with a similar challenge dose of *A. pleuropneumoniae* Δ*aspA* or the *A. pleuropneumoniae* wt (Fig. 6B).

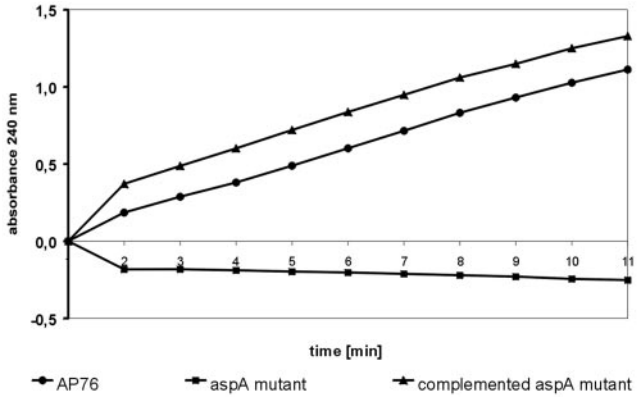

FIG. 4. Aspartase activity in the *A. pleuropneumoniae* wt, *A. pleuropneumoniae* Δ*aspA*, and *A. pleuropneumoniae* Δ*aspA* complemented with an intact *aspA* gene. Active aspartase leads to production of fumarate and an increase of absorbency at 240 nm over time. Cell lysates containing 100 μg of total protein were added to the assay buffer. One of three independent experiments, each performed in triplicate and giving similar results, is shown.

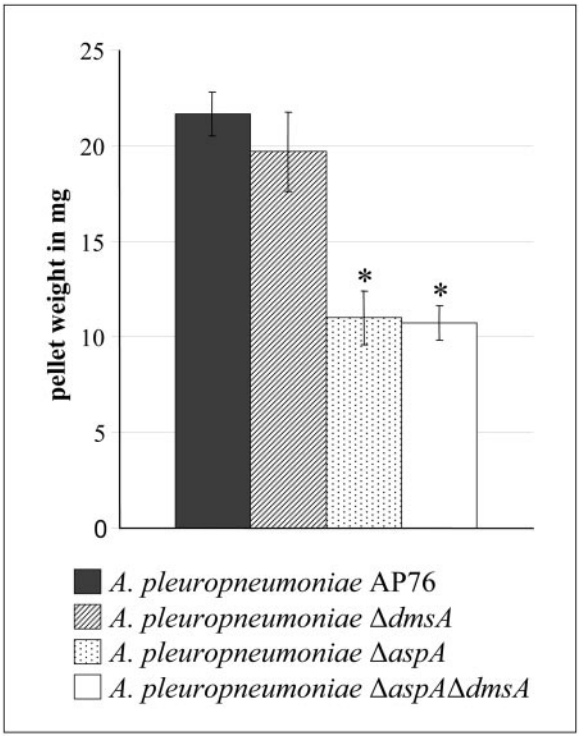

FIG. 5. Anaerobic growth of *A. pleuropneumoniae* AP76 and mutant strains. Bars represent the arithmetic means of dry pellet weights, and hinges represent the standard deviations determined from three distinct experiments. The asterisks indicate statistical significance ($P < 0.01$) as determined by the Student's *t* test.

Serum samples were obtained 1 week before and 1 or 3 weeks after experimental infection. The *A. pleuropneumoniae* wt and both mutants induced a strong humoral immune response, and no significant difference was seen in enzyme-linked immunosorbent assay titers between the different challenge groups (Table 3).

At necropsy, a lower lung lesion score was seen in the groups challenged with either of the two mutant strains compared to groups challenged with the *A. pleuropneumoniae* wt (Table 3), with no apparent differences in the histological examination; *A. pleuropneumoniae* was consistently reisolated from lung lesions

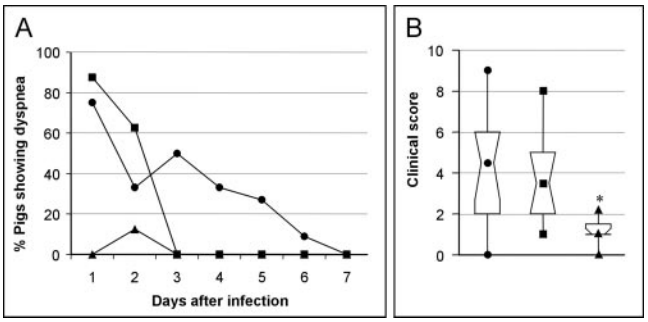

FIG. 6. Clinical symptoms in pigs infected with *A. pleuropneumoniae* wild-type and mutant strains. (A) Occurrence of dyspnea in pigs infected with *A. pleuropneumoniae* wt or mutant strains from days 1 to 7 postinfection. Values are given as the percentage of animals showing dyspnea within groups infected with either the *A. pleuropneumoniae* wt (●), *A. pleuropneumoniae* Δ*aspA* (■), or *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* (▲). (B) Clinical score of pigs infected with different *A. pleuropneumoniae* strains, the *A. pleuropneumoniae* wt (●), *A. pleuropneumoniae* Δ*asp* (■), and *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* (▲). The central symbol in each hourglass shape indicates the geometric mean, the hinges indicate the values in the middle half of the data, and the top and bottom symbols indicate the maximum and minimum values. The asterisk indicates statistical significance ($P < 0.01$) as determined by the Student's *t* test.

in pure culture in 35 of 37 pigs. Reisolation of challenge strains from intact lung succeeded in all animals infected with the high challenge dose of the *A. pleuropneumoniae* wt and *A. pleuropneumoniae* Δ*aspA* but in only two out of seven pigs infected with a comparable dose of *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* (Table 3). Most importantly, only one and six colonies, respectively, could be isolated from these two animals, in contrast to the several hundred *A. pleuropneumoniae* colonies obtained from a surface smear of intact lung from pigs infected with the *A. pleuropneumoniae* wt or with *A. pleuropneumoniae* Δ*aspA*. Surface smears of palatine tonsils, lymph nodes, and heart were sporadically culture positive for *A. pleuropneumoniae* without consistent differences between the challenge groups.

DISCUSSION

The aim of this study was to identify *A. pleuropneumoniae* proteins upregulated in BALF-induced cultures (22), hypothesizing that such proteins would also be upregulated in vivo and might contribute to virulence. Using 2D PAGE in combination with Q-TOF MS, we identified an *A. pleuropneumoniae* aspartate ammonia-lyase to be upregulated by the addition of BALF compared to the addition of saline. The *aspA* gene was further investigated for two reasons: (i) aspartase, like the DMSO reductase previously identified (2), is involved in anaerobic respiration which might be important for persistence of *A. pleuropneumoniae* in the respiratory tract, and (ii) the aspartase of *H. influenzae* had been shown to possess a plasminogen-binding activity which might be related to virulence (41).

The initial experiment, the construction of an isogenic *aspA* in-frame mutation and its complementation in combination with the investigation of aspartase activity in the respective strains, clearly showed that *A. pleuropneumoniae* strain AP76 has a single *aspA* gene. The determination of the transcriptional start site and the presence of a putative FNR binding domain centered at −42.5 implied that *A. pleuropneumoniae aspA*, like *E. coli aspA*, might possess a class II FNR-dependent promoter (25, 51) and that FNR would enhance transcription of aspartase. This hypothesis was supported by the finding that both the amount of aspartase and the aspartase activity in anaerobically grown bacteria are increased compared to aerobic conditions. However, the relative increase of aspartase activity upon anaerobic culture was only twofold in comparison to the aerobic control culture, and induction due to BALF was even less than that.

In order to support the transcriptional activation of *aspA*

TABLE 3. Virulence of *A. pleuropneumoniae* parent and isogenic mutant strains following aerosol challenge

Trial no. and *A. pleuro pneumoniae* challenge strain	Challenge dose (CFU [aerosolized] per 4 pigs)	Time of analysis (day)[a]	Serological response with:		No. of animals with lung lesions/ total no.	Arithmetic mean ± SD of lung lesion score[b,c]	No. of animals with reisolation of *A. pleuropneumoniae* at postmortem analysis/total no.				
			Detergent wash[d]	ApxIIA[c]			Tonsil	Lymph node	Heart	Lung	
										Pneumonic	Intact
Trial 1											
AP76 wt	3.6×10^4	21	7,800 ± 4,480	18.4 ± 15.5	7/8	10.09 ± 10.03	6/8	3/8	4/8	5/6	4/8
AP76 Δ*aspA*	6.7×10^4	21	10,300 ± 11,217[f]	27.9 ± 23.5[e]	4/8[f]	4.90 ± 4.96	6/8	2/8	3/8	4/5	3/8
Trial 2											
AP76 wt	6.76×10^4	7	2,808 ± 2,794	2.5 ± 2.2	7/8	11.38 ± 7.84	7/8	8/8	7/8	7/7	8/8
AP76 Δ*aspA*	16.38×10^4	7	2,400 ± 855	1.9 ± 0.6	8/8	6.89 ± 2.59	7/8	7/8	5/8	8/8	8/8
AP76 Δ*aspA*Δ*dmsA*	11.05×10^4	7	3,400 ± 2,627	2.1 ± 0.8	4/4	6.07 ± 2.05	1/4	3/4	2/4	4/4	1/4
AP76 wt	6.76×10^4	21	38,400 ± 14,780	55.8 ± 12.8	4/4	15.02 ± 11.26	2/4	4/4	1/4	4/4	4/4
AP76 Δ*aspA*Δ*dmsA*	11.05×10^4	21	58,933 ± 57,735	43.3 ± 30.2	3/3	5.70 ± 3.00	3/3	1/3	1/3	3/3	1/3

[a] Day after infection on which animals were sacrificed, postmortem analysis was performed, and antibody titers were determined.
[b] The lung lesion score was determined as described previously by Hannan et al. (21).
[c] Although the lung lesion scores of animals infected with AP76 Δ*aspA* and AP76 Δ*aspA* Δ*dmsA* are lower than those observed for animals infected with the AP76 wild type, this difference is not statistically significant ($P = 0.11$ for AP76 Δ*aspA* and $P = 0.24$ for AP76 Δ*aspA*Δ*dmsA;* Wilcoxon test).
[d] The solid-phase antigen was prepared as described previously (17); the number given is the arithmetic mean of the highest serum dilution resulting in an optical density twice as high as that of the negative control serum at a dilution of 1:100.
[e] Recombinant ApxII was used as a solid-phase antigen as described previously (28); the number given is the arithmetic mean of the serum activity in enzyme-linked immunosorbent assay units.
[f] One animal was euthanized on day 6 after infection due to rectal prolapse. This animal was included in the lung lesion score and reisolation analysis but excluded from determination of serological response.

under anaerobic conditions and upon the induction of BALF, *A. pleuropneumoniae* Δ*aspA*::*luxAB*, a mutant carrying a transcriptional *aspA-luxAB* fusion on the chromosome, was constructed. The luciferase of *P. luminescens* was chosen, as it has been used previously for the performance of gene expression studies in bacteria due to its stability at 37°C and its short half-life (14, 50). In this mutant, no detrimental influences due to copy number are to be expected, as they can occur in plasmid-based systems which have been used previously to investigate promoter function (7). Furthermore, the fusion resulted in an artificial *aspA-luxAB* operon structured similar to the urease (8) and the *tbpBA* operon (46) of *A. pleuropneumoniae* with the stop codon overlapping the Shine-Dalgarno consensus sequence of the downstream gene. This structure was considered to minimize potential polar effects. Using this mutant, a clear-cut transcriptional activation upon anaerobic growth and upon the addition of BALF was observed without the high background seen in aerobic control cultures in the aspartase assay. These findings imply that transcription of *aspA* is upregulated by anaerobiosis and suggest that one component regulating the *A. pleuropneumoniae* aspartase might be HlyX, the *A. pleuropneumoniae* FNR homologue (18). This regulation would resemble the situation in *E. coli*, where *aspA* transcription is likewise upregulated by FNR under anaerobic conditions (25, 51).

However, as induction of AspA activity in the *A. pleuropneumoniae* wt and of luciferase activity in *A. pleuropneumoniae* Δ*aspA*::*luxAB* by BALF was shown upon aerobic growth with shaking, it appears unlikely that decreased oxygen tension alone is responsible for the upregulation of aspartase expression. The suggested influence of other factors coregulating the expression of HlyX-dependent genes is supported by the work of Soltes and MacInnes (42), who showed that HlyX-dependent expression of a *frdA-lacZ* fusion in *E. coli* varied depending on growth phase and carbon source. Therefore, we hypothesize that as-yet-unknown factors in BALF are responsible for upregulation of aspartase activity and transcription of *aspA*. One possibility is that these factors have a HlyX-mediated effect on AspA expression. Furthermore, since the putative FNR binding site upstream of *aspA* (GTGAT-CTAA-ATCAC) also shows high homology with the *E. coli* cyclic AMP receptor protein (CRP)-binding site (12) (AAAT-GTGAT-CTAG-ATCAC-ATTT), regulation by CRP would also seem to be a possibility. However, nothing is known about CRP homologues in *A. pleuropneumoniae*, and also, it cannot be excluded that other transcriptional regulators and promoter structures are involved. Therefore, further studies are needed to elucidate the possible role of HlyX in aspartase regulation and as a global regulator in *A. pleuropneumoniae*.

The reduced growth observed for *A. pleuropneumoniae* Δ*aspA* under anaerobic conditions and the lack of detectable plasminogen-binding activity as has been previously described for *H. influenzae* (41) led us to the hypothesis that the major function of aspartase in *A. pleuropneumoniae* virulence might be the production of fumarate that acts as an electron acceptor for anaerobic respiration, as previously described for *E. coli* (24). This hypothesis was supported by the finding that growth of the mutant was not impaired under aerobic conditions, thereby implying that the role of aspartase in amino acid metabolism is unlikely to be the cause for reduced growth under anaerobic conditions. Since alternative anaerobic respiration pathways are likely to compensate for each other's absence in the presence of suitable substrates, the second *A. pleuropneumoniae* pathway known for anaerobic respiration, which is driven by the DMSO reductase, was deleted by constructing the double mutant *A. pleuropneumoniae* Δ*aspA*Δ*dmsA*. The finding that the growth of this double mutant was indistinguishable from that of the single mutant *A. pleuropneumoniae* Δ*aspA* and our observation that the lack of the *dmsA* gene alone does not diminish growth under anaerobic conditions in vitro (Fig. 4) suggest that *aspA* but not *dmsA* is important for anaerobic growth in vitro.

The challenge of pigs with *A. pleuropneumoniae* Δ*aspA* led to clinical disease with only slightly lower clinical scores than those of pigs challenged with the *A. pleuropneumoniae* wt. The absence of dyspnea from day 3 onwards observed in pigs challenged with *A. pleuropneumoniae* Δ*aspA* is most likely due to the lack of animals with very severe lung lesions as they occurred upon infection with the *A. pleuropneumoniae* wt. Thus, although the lung lesion score did not differ significantly, it was reduced by almost one-half in pigs challenged with *A. pleuropneumoniae* Δ*aspA*.

The clinical score (based on dyspnea, vomitus, and coughing) of pigs challenged with *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* was significantly reduced in comparison to the groups challenged with the *A. pleuropneumoniae* wt or *A. pleuropneumoniae* Δ*aspA*. As clinical symptoms are due primarily to a general colonization of the airways rather than the presence of sequestered tissue, this observation supports the relevance of our finding that *A. pleuropneumoniae* Δ*aspA*Δ*dmsA* could only very sporadically be reisolated from intact lung on days 7 and 21 postinfection. Together, these findings suggest a role for enzymes involved in anaerobic respiration in the colonization of the respiratory epithelium.

The lung score of pigs infected with either one of the mutants was decreased by 50% with respect to the wild type (although the challenge dose for both mutants was approximately double that of the wild type). Despite the lack of statistical significance, this finding is noteworthy, as it strongly supports the attenuation of both strains. Furthermore, it implies that *A. pleuropneumoniae* has additional metabolic pathways to grow and persist within necrotic lesions. Possibly, other enzymes facilitating anaerobic respiration such as the nitrate-trimethylamine oxide reductase (GenBank accession no. NZ_AACK01000004.1) might play a role similar to that described previously in *Mycobacterium bovis* BCG infections (15, 49).

Based on the results of this study, we conclude that enzymes involved in anaerobic respiration play a role in *A. pleuropneumoniae* persistence and virulence. They appear not only to be important for survival in necrotic lesions but, surprisingly, might be required for long-term colonization of intact respiratory epithelium in a presumably aerobic environment.

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